EFFECTS OF VENTILATION, PREMATURITY AND POSTNATAL NUTRITION ON THE DEVELOPMENT OF A NECROTIZING ENTEROCOLITIS-LIKE PHENOTYPE IN PRETERM LAMBS

by

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ABSTRACT

Premature neonates supported by invasive mechanical ventilation (IMV) are at increased risk of necrotizing enterocolitis (NEC). NEC is characterized by disruption to the immature intestinal barrier and enterocyte damage. We previously showed that preterm lambs managed by IMV for 21 days have feeding intolerance, poor weight gain and injury to the distal ileum that resembles NEC. In contrast, preterm lambs managed by non-invasive support (NIS) for 21 days tolerate feedings, have better postnatal growth and better gut histology. The distinct outcomes of preterm lambs managed by IMV and NIS provide an opportunity to evaluate the impact of variables associated with IMV, such as poor postnatal nutrition and sedation, on gut integrity. Using the positive outcome group NIS, preterm lambs received restricted enteral intake (NIS+RN), or sedation (NIS+ES) at levels matched to that of IMV-managed preterm lambs.

The transcription factor PPARγ is required for gut integrity. Transcriptional targets of PPARγ, also important for gut barrier integrity, are FABP2 and the tight junction protein occludin. We hypothesize that in preterm lambs, IMV, NIS+RN and NIS+ES reduce gut integrity in association with decreased ileal expression of PPARγ, FABP2 and occludin.

To test our hypothesis, we studied six groups of preterm lambs: IMV or NIS for 3 or 21 days, and NIS+RN and NIS+ES for 21 days. A reference group was unventilated term lambs. Expression of PPARγ, FABP2 and occludin was measured in ileal tissue.

Preterm lambs managed by IMV and NIS+RN, had impaired postnatal growth and reduced expression of PPARγ and FABP2 compared to preterm lambs managed by NIS. Preterm lambs managed by NIS+ES did not have feeding intolerance or poor postnatal growth, but did have reduced PPARγ and FABP2 expression compared to preterm lambs managed by NIS. Contrary to our hypothesis, occludin protein abundance was not impacted by mode of ventilation, nutrition or sedation.

In conclusion, IMV, NIS+RN and NIS-ES decreased PPARγ and FABP2 expression in the preterm lamb ileum. Disruption of PPARγ and FABP2 expression by IMV and inadequate nutrition may result in detrimental effects on gut integrity and contribute to the observed NEC-like phenotype in preterm lambs.

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INTRODUCTION

Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC), a severe gastrointestinal disease, is one of the most common life-threatening diseases affecting neonates[1, 2]. The majority of NEC cases occur in premature infants less than 37 weeks gestation, with a mortality rate of 20-50%[2]. Typical onset of NEC is in the first three months of life after feeding intolerances are observed[2]. There are three stages of NEC in the Bell's diagnosis criteria. In stage I and II NEC, symptoms include abdominal distention, bloody stool, absent bowel sounds and ascites[2, 3]. In more advance Bell's stage III NEC, bacterial translocation, multiple dilated loops of the small bowel, gas-filled loops, bowel perforation and septic shock occur[2, 3]. About one third of NEC cases require surgery[3]. Long-term consequences of NEC include prolonged hospitalization, short bowel syndrome, parenteral nutrition-associated cholestasis, significantly impaired growth and poor long-term neurodevelopment[4].

Despite ongoing research, the pathogenesis, prevention and treatment of NEC is not completely understood. The main factors thought to be involved in the development of NEC are prematurity, ischemic injury, early formula feeding, dysbiosis, intestinal permeability and inflammatory responses[1, 2]. One component of NEC is damage to the enterocytes that comprise the intestinal epithelium, and subsequent loss of integrity of the intestinal barrier. Damage and loss of integrity of the intestinal epithelium in NEC (see Figure 1) is severe and results in in increased gut permeability and bacterial translocation.

Prematurity further compromises the gastrointestinal integrity. Prematurity is marked by immaturity of gut motility and intestinal epithelial cells, increasing the amount of bacteria within the gut and reducing the gut's ability to protect against bacterial translocation[2]. Intestinal permeability further increases the potential for bacteria to translocate, leading to systemic inflammatory responses[1, 2].

Respiratory Support and NEC

While risk factors for NEC are many, one important risk factor for NEC in preterm infants is invasive mechanical ventilation (IMV). Preterm neonates frequently require IMV for respiratory support secondary to immature lung development. In IMV, an endotracheal tube is placed to facilitate ventilation. Infants with IMV require greater sedation and analgesics to minimize discomfort and stress[7, 8]. Infants who required IMV during their neonatal ICU course are approximately 13 times more likely to develop NEC than preterm infants who do not require IMV[4]. Furthermore, inflammation that accompanies NEC increases metabolic demands, causing respiratory decompensation and an increased need for mechanical ventilation[9]. While we know there is a link between prematurity, IMV and NEC, the molecular mechanisms that give rise to NEC are not completely understood.

Signaling Pathways Important for Gut Integrity

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nutrient sensitive nuclear receptor that modulates gene transcription. PPARγ is expressed in a number of tissues, including the epithelium of the small and large intestines. In animal models,

pretreatment with a PPARγ agonist has been shown to protect against NEC-like injuries to enterocytes[10, 11, 12]. One way that agonists of PPARγ ameliorate gastrointestinal injury is by promoting and stabilizing epithelial differentiation[13].

Activation of PPARγ, in the intestines, is reliant on intestinal fatty acid binding protein or FABP2. FABP2 has a high affinity for binding long-chain saturated and unsaturated fatty acids[14]. The role of FABP2 is to transport long chain fatty acids from the cytosol into the nucleus of the enterocyte. They can then act as ligands for PPARγ, thus enhancing transcriptional activity[15].

Transcriptional target genes of PPARγ include those important for enterocyte development and the intestinal barrier, including FABP2 itself. FABP2 is located in the cytosol of the tips of microvilli and only expressed by mature enterocytes[16]. Animal and human studies have shown that upon mucosal damage, enterocytes release FABP2 into circulation[10, 16, 17, 18, 19]. Intestinal FABP may be a useful plasma and urinary marker for enterocyte integrity and development of NEC[16, 18, 19, 20]. There is a positive association between circulating FABP2 concentrations and severity of NEC[2, 19, 20]. Interestingly, pretreatment with either fish oil or PPARγ agonist 15d-PGJ_2 reduces the redistribution of FABP2 from mucosa into the circulation following induced ischemia and reperfusion in the rat[10]. This suggests a protective relationship between PPARγ and FABP2.

One mechanism by which PPARγ and FABP2 may confer gut protection is via maintenance of tight junctions and reduced gut permeability. Preterm infants diagnosed with NEC have decreased tight junction barrier function and decreased expression of the protein occludin[21]. Occludin's role in tight junctions is to regulate epithelial barrier function[21, 22]. Occludin is also required for cells to transduce cytokine-mediated signals

that either increase the epithelial barrier or decrease the large solute barrier[22]. Without occludin, there is a complete loss of tight junction barrier function that results in elevated permeability, mucosal injury and inflammation. Active PPARγ can bind to the promoter region of the occludin gene, directly raising occludin expression[21]. A pretreatment of either PPARγ agonist, 15d-PGJ_2 or fish oil, has been shown to protect the morphology of tight junctions and occludin expression in the rat[10]. Permeability is an important predisposing factor involved in the development of NEC in humans, therefore modulation of barrier function could impact NEC outcomes in preterm infants.

Preliminary Data

Our lamb model is designed to examine the mechanisms of preterm birth and chronic ventilation on disease development. Our lamb model of preterm birth is unique, follows neonatal intensive care unit protocols and is clinically relevant. Clinical relevance of our model is further enhanced by the developmental biology of the lamb gut closely resembling that of human neonates[17]. Details of the lamb model are further discussed in Methods.

Our preliminary data show mode of ventilation affects disease outcomes in preterm lambs. Our lab showed preterm lambs managed by IMV for 21 days have feeding intolerance, poor weight gain and significant injury to the distal ileum that resembles NEC[23,24] (see Table 1, Figure 2 and 3). In contrast, preterm lambs managed by non-invasive support (NIS) for 21 days tolerate feedings that led to larger enteral intake, better postnatal growth and better gut histology[24]. Morphometric analysis of gut histology shows that in preterm lambs managed by IMV, villus length and crypt depth is decreased

both at 3 days and 21 days compared to gestationally matched, preterm NIS lambs. Apoptosis, programmed cell death, is a regulated process that ensures gut integrity. Apoptosis was significantly reduced in the ileum of preterm lambs managed by IMV compared to preterm lambs managed by NIS. These data suggest IMV preterm lambs experience gut necrosis. Lastly, within the IMV group of preterm lambs, two lambs had damage severe enough to meet the criteria of stage III NEC diagnosis[25].

The distinct outcomes of preterm lambs managed by IMV and NIS provide an opportunity to evaluate the impact of different variables associated with IMV and NIS, such as poor postnatal nutrition and growth, and sedation on gut integrity. Our lab has previously examined the effect of isolated postnatal nutrition and poor growth, and isolated sedation on alveolar formation[26]. We used the positive outcome mode of ventilation, NIS and manipulated both postnatal nutrition and sedation. As previously mentioned, preterm lambs managed by IMV for 21 days have feeding intolerance and poor growth relative to preterm lambs managed by NIS. Additionally, preterm lambs managed by IMV require sedation, whereas preterm lambs managed by NIS do not. Therefore, we used preterm lambs managed by NIS with restricted nutrition (NIS+RN group) or standard nutrition (NIS control group) to assess the isolated contribution of restricted nutrition to gut integrity. The NIS+RN group was defined as preterm lambs managed by NIS with nutrient intake restricted to that tolerated by IMV lambs. A group of preterm lambs managed by NIS receiving excess sedation (NIS+ES group) were also compared to preterm lambs managed by NIS without sedation (NIS control group). NIS+ES group was defined as preterm lambs managed by NIS that received pentobarbital dosage matched to that historically needed by IMV lambs. The contribution of NIS+RN

and NIS+ES need to be evaluated to further understand the roles of postnatal nutrition and sedation in signaling pathways important to gut integrity.

Study Aims

Our preliminary data show that preterm lambs managed by IMV have a NEC-like phenotype with significant enterocyte damage, and feeding intolerance. However, the effects of prematurity, mode of ventilation, postnatal nutrition and sedation on PPARγ, FABP2 and occludin expression in the distal ileum remain unknown.

We hypothesize that in preterm lambs, IMV reduces gut integrity in association with decreased ileal expression of PPARγ, FABP2 and occludin. Furthermore, we hypothesize that NIS+RN and NIS+ES will also decrease ileal expression of PPARγ, FABP2 and occludin.

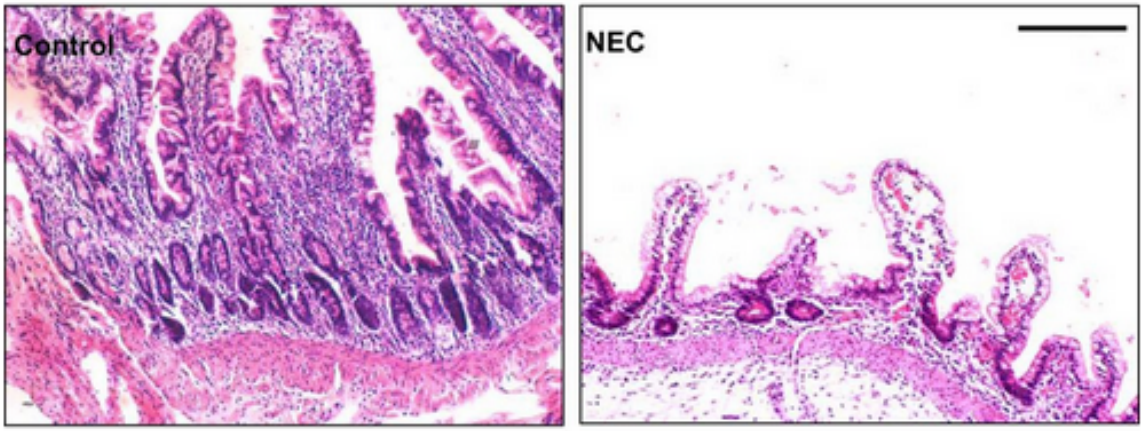

Figure 1. Histological appearance of terminal ileum of preterm infant without and with NEC[5]

Intestinal epithelium of the small intestines is made up of villi that consist of microvilli projecting from enterocytes. Enterocytes are responsible for absorbing nutrients and contain digestive enzymes. Between the villi are pits of crypts. Crypts are responsible for the proliferation of intestinal epithelial cells, enterocytes and goblet cells[6]. Crypt depth is defined as the depth of the invagination between adjacent villi. Damage and loss of integrity of the intestinal epithelium in NEC (*right*) is severe compared to preterm infant without NEC (*left*). In NEC, villi are less in number and deteriorated, and crypt depths appear greater.

Table 1. Weight gain of preterm lambs managed by IMV and NIS for 21 days[23]

	Preterm 21 days	
	NIS	IMV
Weight at delivery (Kg)	4.1 ± 0.9	4.5 ± 0.5
Weight at end of study (Kg)	4.5 ± 0.4*	3.9 ± 0.4

Preterm lambs managed by NIS gain significantly more weight (mean± SD) than preterm lambs managed by IMV (n=4 each, *$p < 0.05$).

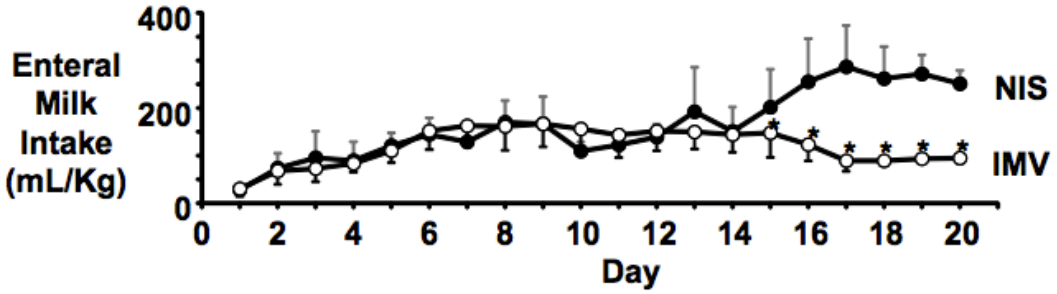

Figure 2. Feeding volume of preterm lambs managed by IMV and NIS over a 21 day study period[24]

Daily intake in mL/Kg is depicted on the y-axis and day of life on the x-axis. Preterm lambs managed by IMV (white circles) consumed significantly less enteral intake compared to NIS preterm lambs (black circles) during the third week of life (n=4 each, *p < 0.05).

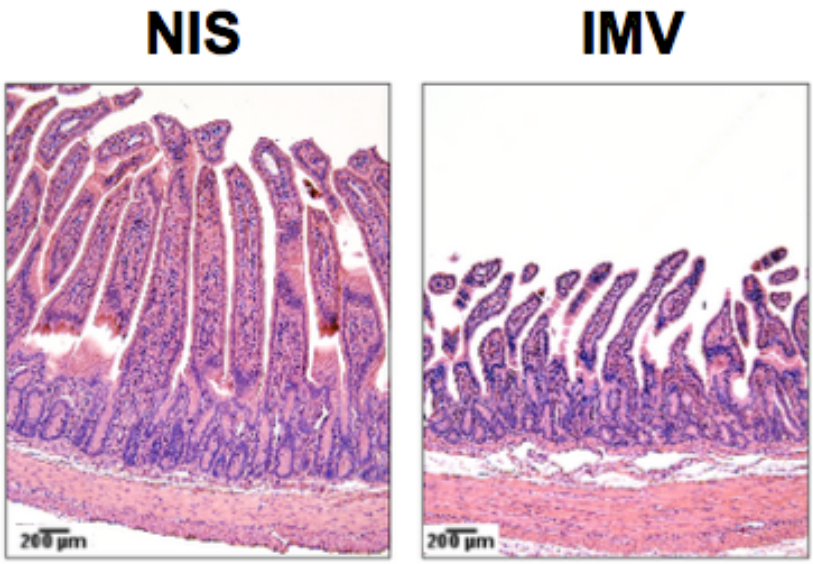

Figure 3. Histological appearance of distal ileum of preterm lamb managed by NIS and IMV for 21 days

Villi in preterm lambs managed by IMV (*right*) appear fragmented with greater deterioration and crypt depth compared to NIS managed preterm lambs (*left*).

METHODS

We have extensive experience with our preterm lamb model[26, 27, 28]. Lambs were delivered at 132 ± 2 days; term equivalent age is 150 days gestation. This is comparable to 28-37 week gestational age in humans[17]. Ewes were treated with dexamethasone phosphate, ~36 h before operative delivery. All preterm lambs were initially intubated and received IMV for the first three hours. Preterm lambs in the IMV group continued invasive ventilation method for 3 or 21 days. Preterm lambs in the NIS group were then weaned to a high-frequency, flow-interruption ventilator for 3 or 21 days. We chose to examine lambs at 3 days to assess acute effects, and 21 days to assess chronic effects. The preterm lambs are term-equivalent at 21 days. We also used term-born lambs as a comparison group (NL-1d). All lambs received enteral feedings orogastrically. For the first 3 days of life, lambs received ewe's colostrum formula as tolerated (Kid & Lamb Colostrum Replacement, Land O Lakes, Arden Hills, MN). At day 4 of life, colostrum was replaced with mature ewe's formula (Sav-A-Lam Milk Products, Chilton, WI). Volume of the feedings were gradually increased by 3-5 ml increments, as tolerated, to attain a goal of 60-80 kcal/kg/d. Enteral feedings continued in IMV lambs due to sedation; however, NIS lambs were able to take milk from a bottle at about 4-5 days of life. Feeding intolerance was indicated by residuals in the stomach 30 minutes after each feeding. Lambs with apparent gut pathophysiology had an abdominal X-ray for detection of gas-filled loops. Feeding and growth data were collected continuously over each 24-

hour period.

Feeding tolerance and weight gain is impacted significantly by the mode of ventilation IMV (see Table 1 and Figure 2). In evaluating the components of IMV, restricted nutrition and sedation, total calories and weight gain were measured. At 21 days, NIS+RN preterm lambs had gain significantly less weight than the preterm lambs managed by NIS ($p \leq 0.05$). Consistent with decreased weight gain, NIS+RN lambs received fewer total calories, fluid, protein and fat than NIS. At 21 days, NIS+ES preterm lambs had similar weight gain, as well as total calories, fluid, protein and fat intake as preterm lambs managed by NIS[26].

Samples of the last 2 cm of the distal ileum were collected at necropsy at 3 days, 21 days or 24 hours after term birth, and flash frozen.

For the purpose of examining different methods of chronic ventilation on NEC-like phenotype, ileal tissue* samples were analyzed in IMV and NIS lambs at postnatal 3 day and 21 day, n=5/group, and term lamb (NL-1d), n=4/group. To examine the effect of postnatal nutrition and sedation on NEC-like phenotype study groups, NIS+RN and NIS+ES were included. Restricted nutrition preterm lambs were managed by NIS for 21 days and were fed a reduced volume of milk based on the volume (mL/Kg/day) historically tolerated by preterm lambs managed by IMV for 21 days (see Figure 2), n=4/group. NIS+RN lambs received less total calories, protein and fat than control NIS lambs, n=5/group. All preterm lamb groups received pentobarbital (Abbott Laboratories, North Chicago, IL) at 1 to 2 mg/Kg, as needed (heart rate >200 beats/min). The NIS and NIS+RN groups received pentobarbital at 0.8±0.6 mg/Kg/day. The NIS+ES group

* Ileal tissue is examined as NEC largely affects the distal ilium[2].

received pentobarbital at 4.8 mg/Kg/day. This dose is based on daily dosage (mg/Kg/day) historically needed to keep preterm lambs supported by IMV comfortable and calm[23, 29].

Real-Time PCR

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyze mRNA transcript level of FABP2 in the IMV and NIS at 3 days and 21 days and term NL-1d. RNA was extracted from ileal tissues and cDNA was generated. GAPDH was used as an internal control.

Western Blot

Protein abundance of PPARγ, FABP2 and occludin was analyzed in all preterm lamb study groups (IMV and NIS 3 days and 21 days, NL-1d, NIS+RN and NIS+ES) and term reference lambs using Western blot. Lamb ileal tissue samples were homogenized and insoluble material and fat were removed by centrifugation. Equal amounts of total protein from tissue 10 μg, were loaded into each well on a 10% Bis-Tris Midi Gel, 1.0 mm x 26 well, and transferred to membrane. Detection of PPARγ protein abundance (58 kDa) was accomplished by primary antibody PPARγ (1:400 dilution: #sc-7196, Santa Cruz) followed by a secondary antibody rabbit IgG (1:8000 dilution #7074S, Cell Signaling Technologies). Detection of FABP2 protein abundance (14-15 kDa) was accomplished by incubation of membrane with primary antibody FABP2 (1:100 dilution: intestinal FABP #PA1-40304, Invitrogen) followed by secondary antibody rabbit IgG overnight. Detection of occludin protein abundance (65 kDa) was accomplished by primary antibody occludin monoclonal (1:1000 dilution #33-1500, ThermoFisher

Scientific) secondary antibody mouse IgG (1:3000 dilution #7076S, Cell Signaling Technologies). GAPDH (1:6000 dilution #2118S, Cell Signaling Technologies) was used as an internal control for all antibodies.

Immunofluorescence

Indirect immunofluorescence was used on ileal tissue sections of preterm lambs managed by IMV, NIS, NIS+RN and NIS+ES preterm lambs for 21 days to localize FABP2 within the ileum. Primary antibody of intestinal FABP (1:1500 dilution #ab60272, Abcam) was applied and incubated overnight. Antibodies were then stained with a fluorescein-labeled antibody, SA-AF555 (1:500 dilution).

Statistical Analysis

Based on our experience, sample sizes of 4-6 are sufficient to determine significant differences between preterm lambs managed by IMV, NIS and NIS+RN and NIS+ES[24, 25, 26]. All results are expressed as mean ± standard deviation (SD). Statistical comparisons for mRNA and protein abundance between groups are made using ANOVA analysis, with fishers least squared *post hoc* test using Statview 5 software package (SAS Institute, Inc.). A statistically significant difference between groups is determined with a p-value of < 0.05.

RESULTS

PPARγ Protein Abundance

Based on of the importance of PPARγ to epithelial cell integrity, we measured PPARγ protein abundance. Figure 4 shows PPARγ abundance is less in preterm lambs managed by IMV for 3 days, compared to preterm lambs managed by NIS ($p \leq 0.05$). In addition, immunohistochemistry staining of the distal ileum demonstrates that PPARγ localizes to the villi of preterm lambs, and appears less in preterm lambs managed by IMV compared to preterm labs managed by NIS (see Figure 5).

PPARγ protein abundance was also evaluated in preterm lambs managed by IMV and NIS for 21 days (see Figure 6). Preterm lambs managed by IMV for 21 days had significant reduction in ileal PPARγ protein abundance compared to preterm lambs managed by NIS ($p \leq 0.05$). Preterm lambs managed by NIS+RN had significantly less PPARγ protein abundance compared to preterm lambs managed by NIS (see Figure 6; $p \leq 0.05$). PPARγ protein abundance in NIS+RN was similar to PPARγ protein abundance in preterm lambs managed by IMV for 21 days ($p=0.626$). PPARγ protein abundance of preterm lambs managed by NIS+ES was less than preterm lambs managed by NIS, although not statistically significant (see Figure 6; $p=0.092$). There was no statistically significant difference between the non-ventilated term birth lambs and any of the preterm lamb groups.

FABP2 Expression

FABP2 was measured due to its role in the activation of PPARγ, as well as being a transcriptional target of PPARγ. To understand the effects on FABP2 transcription, FABP2 mRNA was measured in the distal ileum of preterm lambs managed by IMV and NIS for 3 days and 21 days and term lambs. FABP2 mRNA was significantly less in preterm lambs managed by IMV for 3 days and 21 days compared to preterm lambs managed by NIS for 3 days and 21 days (see Figure 7 and 8; $p \leq 0.05$). Preterm lambs managed by IMV had decreased FABP2 mRNA levels compared to term lambs ($p \leq 0.05$). However, preterm lambs managed by NIS did not significantly alter FABP2 mRNA levels compared to term lambs ($p=0.36$).

To understand the functional effects, FABP2 protein abundance was also measured in all groups. Preterm lambs managed by IMV for 3 days had reduced FABP2 protein abundance compared to preterm lambs managed by NIS for 3 days (see Figure 7; $p \leq 0.001$). FABP2 protein abundance in preterm lambs managed IMV remained significantly less compared to preterm lambs managed by NIS for 21 days (see Figure 8; $p \leq 0.05$). However, by 21 days, the reduction in FABP2 protein abundance in preterm lambs managed by IMV was not statistically significant compared to term lambs ($p=0.103$).

Postnatal nutrition had a significant impact on FABP2 protein abundance (see Figure 9). Preterm lambs managed by NIS+RN for 21 days have a significant reduction in FABP2 protein abundance compared to preterm lambs managed by NIS for 21 days ($p \leq 0.05$). In fact, preterm lambs managed by NIS+RN had similar FABP2 protein abundance as preterm lambs managed by IMV for 21 days ($p=0.702$). Despite similar

postnatal growth, as preterm lambs managed by NIS for 21 days, preterm lambs managed by NIS+ES had significantly less FABP2 protein abundance than NIS managed preterm lambs ($p \leq 0.05$). Preterm lambs managed by NIS+ES had similar FABP2 protein abundance as preterm lambs managed by IMV for 21 days ($p=0.73$).

Occludin Protein Abundance

Occludin, a transcriptional target of PPARγ, is important for maintaining tight junction function. In contrast to FABP2 protein abundance, preterm lambs managed by IMV for 3 days had greater occludin protein abundance compared to preterm lambs managed by NIS for 3 days (see Figure 10; $p<0.05$).

At 21 days, there was no statistical significant difference between preterm lambs managed by IMV, NIS+RN or NIS+ES and preterm lambs managed by NIS (see Figure 11). Additionally, there was no significant difference between any preterm lamb groups and term lambs.

FABP2 Localization Immunofluorescence

Immunofluorescence images of the distal ileum of preterm lambs, managed for 21days by IMV, NIS, NIS+RN and NIS+ES (see Figure 12), show localization of FABP2 in the cytoplasm of the enterocytes. NIS appears to have greater localization of FABP2 in the enterocytes compared to IMV. Additionally, it appears that the villus morphology is impacted in preterm lambs managed by IMV. Villi appear to be fragmented, shorter in length and less in abundant compared to NIS. Preterm lambs managed by NIS+RN appear to have similar localization of FABP2 and villi morphology as preterm lambs

managed by IMV. In contrast, preterm lambs managed by NIS+ES appear to have greater localization of FABP2 and the morphology of the villi appear less injured compared to preterm lambs managed by IMV.

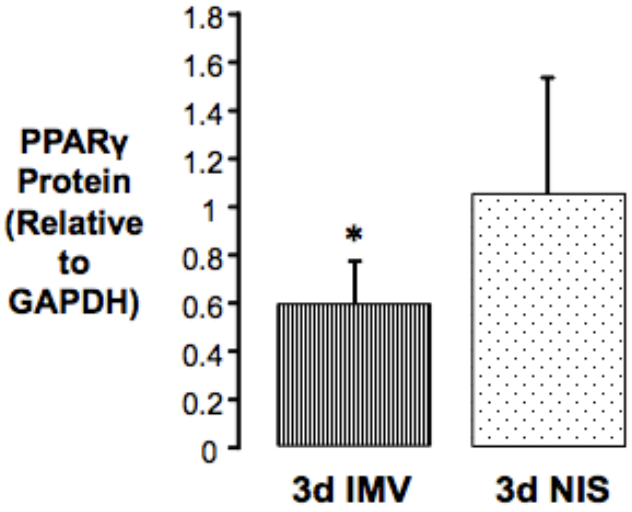

Figure 4. PPARγ protein abundance preterm lambs managed by IMV and NIS for 3 days
Preterm lambs managed by IMV for 3 days have a significantly reduced ileal PPARγ protein abundance relative to preterm lambs managed by NIS (n=5/group, $^{*}p \leq 0.05$).

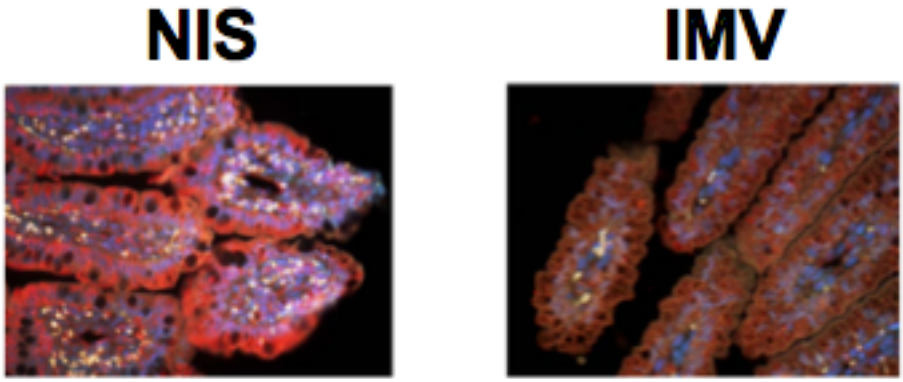

Figure 5. Immunohistochemistry localization of PPARγ in villi of preterm lambs managed by NIS and IMV for 3 days
PPARγ is shown in white. PPARγ appears to be less in the distal ileum of preterm lambs managed by IMV (*left*) than in NIS managed preterm lambs (*right*).

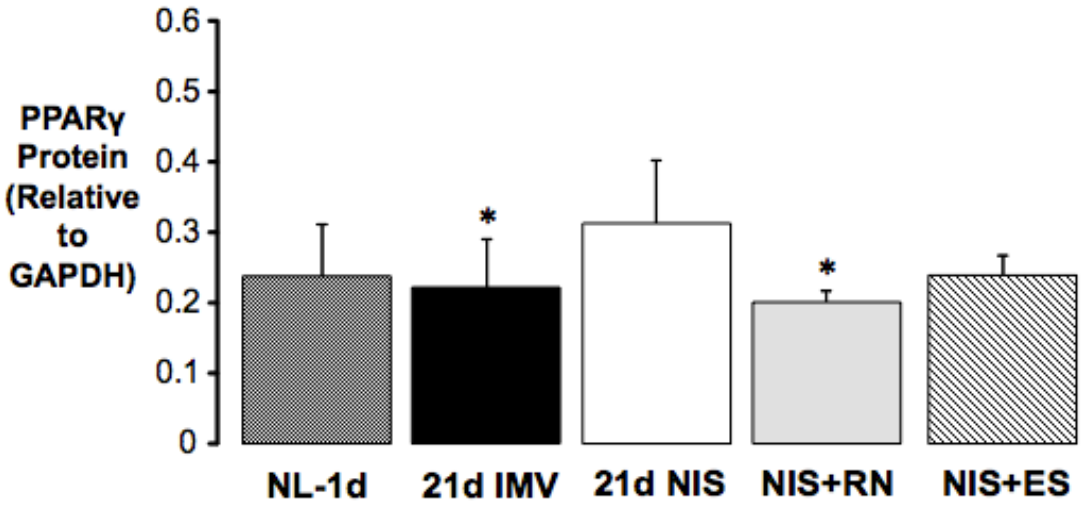

Figure 6. PPARγ protein abundance in distal ileal tissue in term lambs and preterm lambs managed by IMV, NIS, NIS+RN and NIS+ES for 21 days

Preterm lambs managed by IMV and NIS+RN have significantly less PPARγ protein abundance than preterm lambs managed by NIS (n=4-5/group; *p<0.05 compared to 21d NIS).

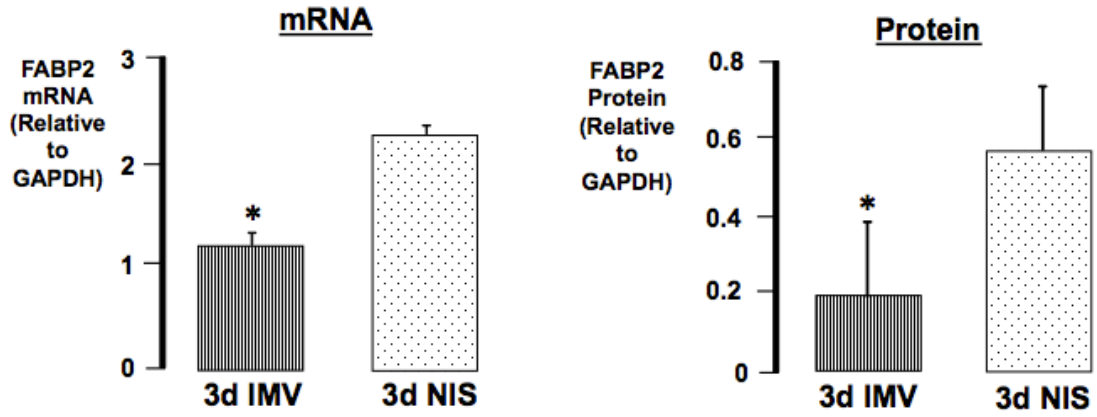

Figure 7. FABP2 mRNA and protein abundance in distal ileal tissue of preterm lambs managed by IMV compared to NIS preterm lambs for 3 days

FABP2 mRNA and protein abundance in preterm lambs managed by IMV for 3 days is significantly lower compared to preterm lambs managed by NIS for 3 days (n=3-5/group; *p < 0.05).

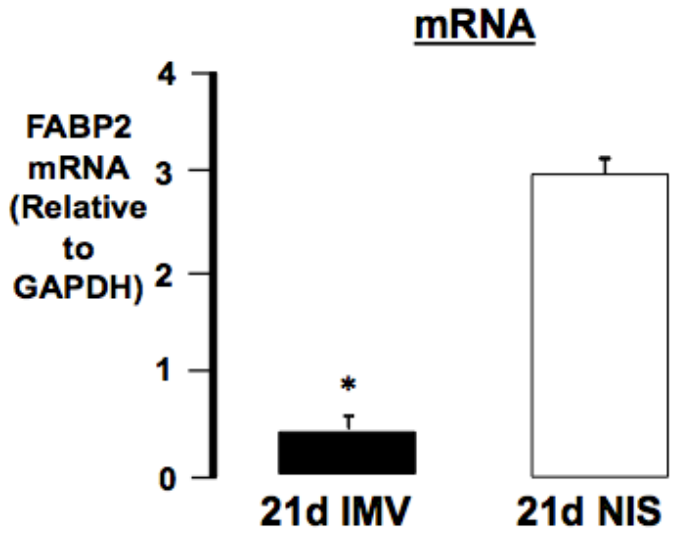

Figure 8. FABP2 mRNA in distal ileal tissue of preterm lambs managed by IMV compared to NIS preterm lambs for 21 days

FABP2 mRNA and protein abundance in preterm lambs managed by IMV for 21 days is significantly lower compared to preterm lambs managed by NIS for 21 days (n=3-5/group; *p < 0.05).

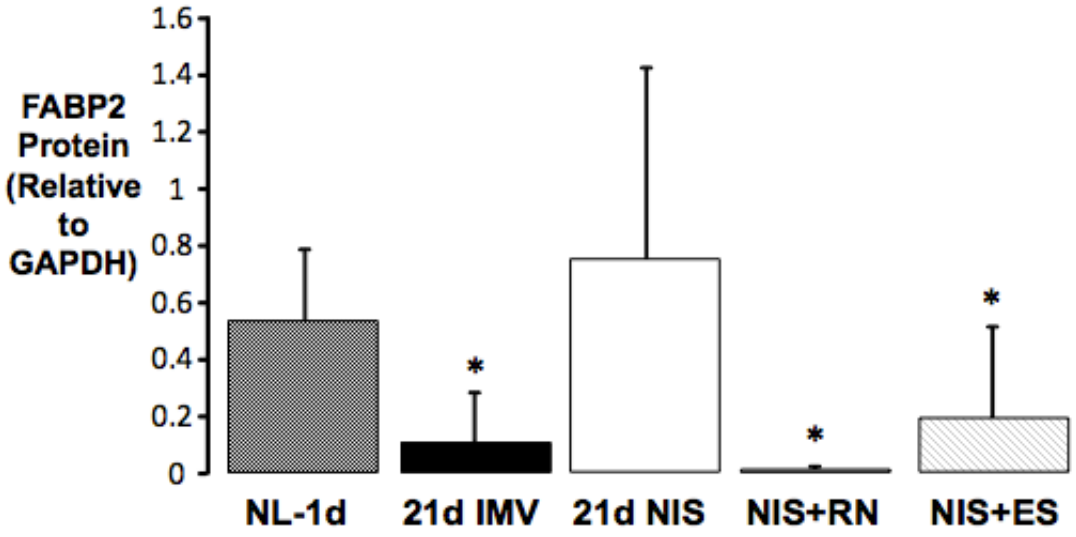

Figure 9. FABP2 protein abundance in distal ileal tissue in term lambs and preterm lambs managed by IMV, NIS, NIS+RN and NIS+ES for 21 days

Preterm lambs managed by IMV, NIS+RN and NIS+ES for 21 days have a significant reduction in FABP2 protein abundance than preterm lambs managed by NIS for 21 days (n=4-5/group; *p<0.05 compared to 21d NIS).

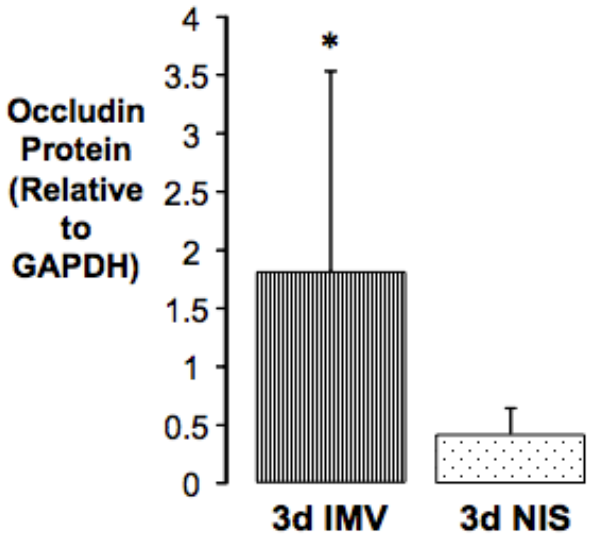

Figure 10. Occludin protein abundance in distal ileal tissue in preterm lambs managed by IMV and NIS for 3 days

Preterm lambs managed by IMV for 3 days had significantly more ileal occludin protein abundance compared to preterm lambs managed by NIS for 3 days (n=5/group; *p<0.05).

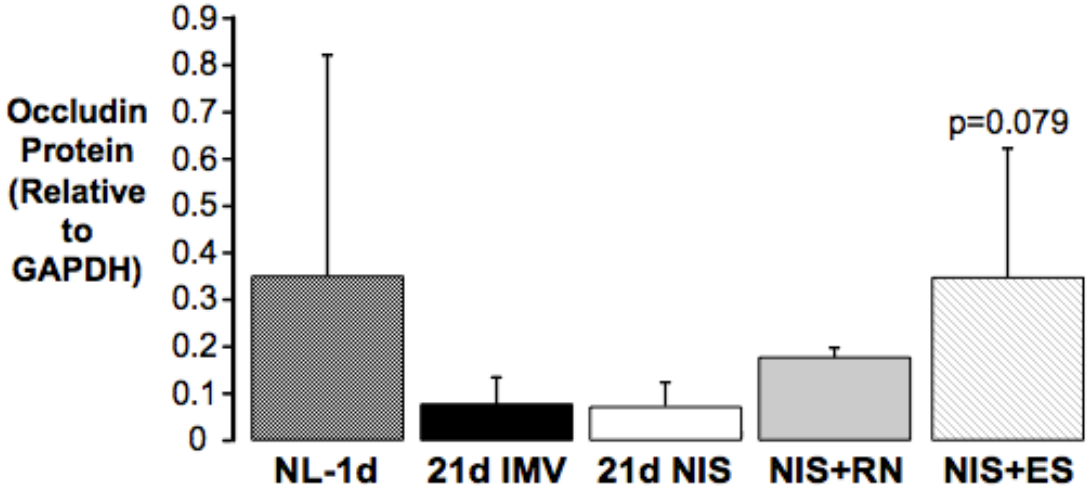

Figure 11. Occludin protein abundance in distal ileal tissue in term lambs and preterm lambs managed by IMV, NIS, NIS+RN and NIS+ES for 21 days

There was no significant difference in occludin protein abundance between groups. p= 0.079 compared to preterm lambs managed by NIS for 21 days.

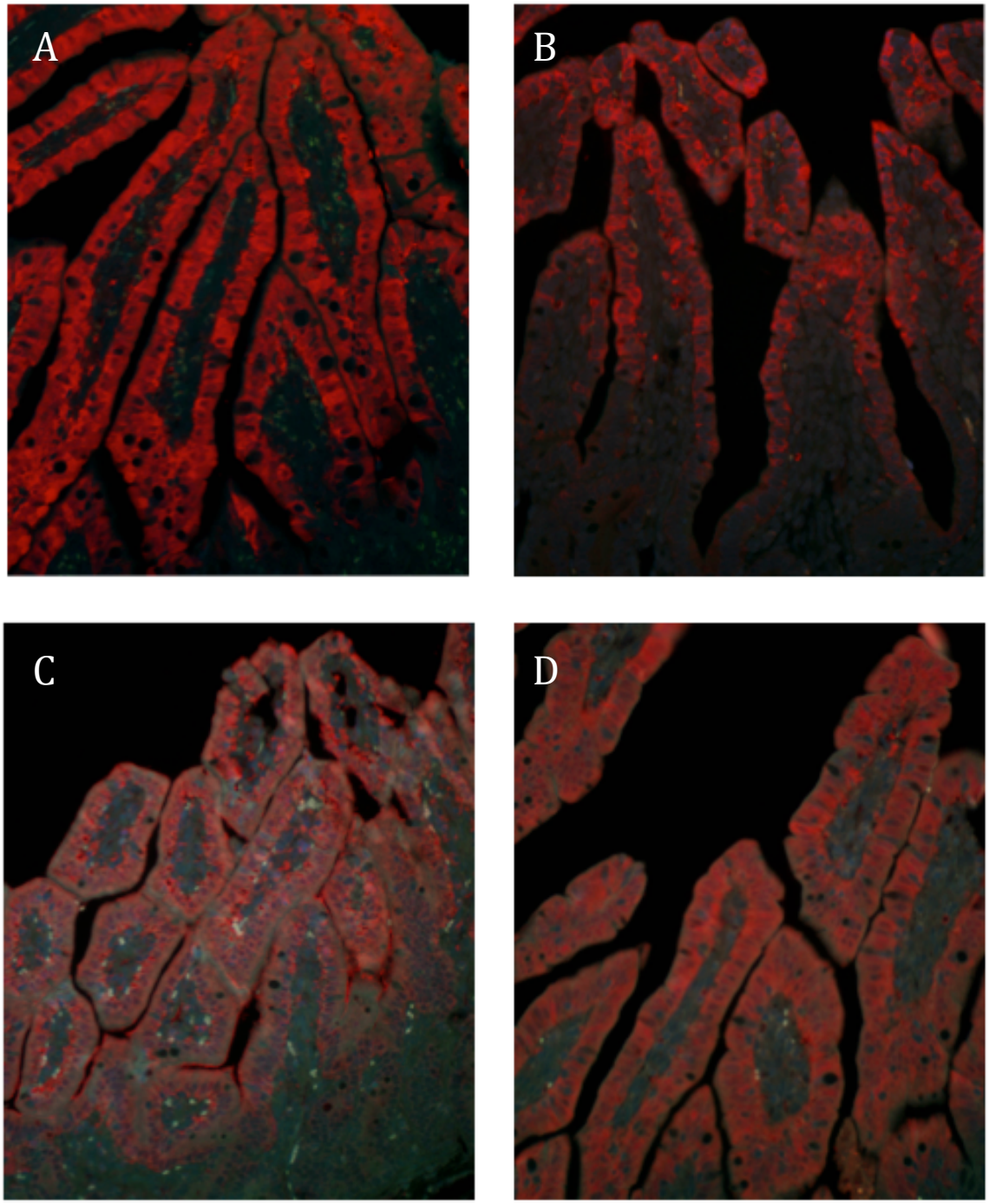

Figure 12. Localization of FABP2 in distal ileum of preterm lambs managed by NIS, IMV, NIS+RN and NIS+ES for 21 days

All images are at the same magnification. Red stain is FABP2. Immunofluorescence was not used for quantification in this stage of the experiment. Order of images: NIS (A), IMV (B), NIS+RN (C) and NIS+ES (D).

DISCUSSION

Understanding molecular mechanisms of gut integrity in response to prematurity and mode of ventilation is important to understanding the pathogenesis of NEC. The evaluation of preterm lambs managed by IMV and NIS for 3 days and 21 days supports a more invasive form of ventilation and significantly alters the mucosal barrier morphology as well as PPARγ and FABP2 expression, also important to gut integrity. Decreased expression of PPARγ and FABP2 may indicate an interruption in the PPARγ-FABP2 signaling pathway, and subsequent disruption of gut integrity. Contrary to our hypothesis, neither IMV nor NIS effected occludin expression. These data suggest that occludin-regulated mucosal barrier function is not a mechanism by which IMV and postnatal nutrition give rise to a NEC-like phenotype.

In preterm lambs managed by NIS, restricted nutrition decreased weight gain, altered villi morphology and PPARγ and FABP2 expression similar to preterm lambs managed by IMV. However, preterm lambs managed by NIS plus excessive sedation did not alter villi morphology or have a significant impairment of the PPARγ and FABP2 expression compared to preterm lambs managed by IMV. Occludin protein abundance was not impacted by mode of ventilation and does not appear to be associated with PPARγ expression.

In epidemiological studies, prematurity is the only factor to be a consistent independent determinant of NEC[30]. Intestinal immaturity is characterized by immature

motility, digestion, absorption, immune defenses and barrier function[3]. However, non-ventilated term born lambs did not differ significantly in PPARγ protein abundance, FABP2 expression or occludin protein abundance compared to any of our premature ventilated IMV groups or NIS groups. Within the term lambs selected for this study, protein abundance measurements had large variability. This variability likely reflects healthy biological variability.

We hypothesized PPARγ protein abundance would be reduced with gastrointestinal injury seen in preterm lambs managed by IMV. PPARγ protein abundance was reduced both at 3 days and 21 days in preterm lambs managed by IMV. Our observation of reduced PPARγ abundance in preterm lambs managed by IMV is consistent with other animal models of induced intestinal damage[11]. Our lab has previously shown that preterm lambs managed by NIS+RN had significant damage to lung formation and reduced lung PPARγ protein abundance relative to that seen in IMV lambs[26]. Our study found PPARγ protein abundance to be reduced in preterm lambs managed by NIS+RN for 21 days. This decrease in PPARγ activity in the lung and in the distal ileum suggests a decrease in availability of a PPARγ agonist.

An important agonist for PPARγ is the long chain polyunsaturated fatty acid, DHA. Restricted feeding, secondary to prematurity, is linked to marked differences in available essential fatty acids in preterm neonate[31, 32]. In our lamb model, preterm lambs managed by IMV for 21 days have altered circulating fatty acid profiles compared to term born lambs[33]. Our results suggest that feeding intolerance in preterm lambs managed by IMV and caloric restriction in NIS+RN limits fatty acid substrate availability and thus reduces PPARγ activity.

Limited PPARγ activity results in a decrease expression and activity of its downstream target FABP2. FABP2 was decreased both at the transcriptional level and at the protein level in both preterm lambs managed by IMV and preterm lambs managed by NIS+RN. The reduction of FABP2 expression is supported by the appearance of a reduced localization of FABP2 in the enterocytes of preterm lambs managed by IMV and NIS+RN compared to NIS at 21 days. A reduction of FABP2 reduces the shuttle of fatty acids to the nucleus, subsequently inactivating PPARγ and interfering with the PPARγ-FABP2 feedback loop. The reduction of both PPARγ protein abundance and FABP2 expression supports the protective relationship PPARγ and FABP2 have on gut integrity.

Based on animal[10] and human[21] studies of NEC, we hypothesized that occludin protein abundance would also be reduced by a decrease in PPARγ activity. In contrast to PPARγ protein abundance and FABP2 expression, occludin protein abundance was greater in preterm lambs managed by IMV for 3 days compared to preterm lambs managed by NIS. Based on greater PPARγ levels in preterm lambs managed by NIS at 21 days, we expected a biological priority of PPARγ would be to promote the transcription of occludin to mediate the paracellular permeability common in prematurity. Introduction of PPARγ agonist has been shown to reduce permeability and tighten the junction function[34]. Protein abundance may not be the appropriate assessment of occludin transcription. Occludin functions in tight junctions with claudin to form the sealing element[21] and may be bound too tightly for protein to be assessed. Occludin mRNA has been shown to be markedly decreased in NEC tissue in neonates[21]. Occludin mRNA should be assessed in our lamb model to confirm or elucidate our protein results.

We hypothesized excessive sedation would have a significant impact on gut

integrity as sedation affects gastrointestinal motility, absorption and blood flow[35-38]. Preterm lambs managed by NIS+ES had caloric intake and growth similar to preterm lambs managed by NIS. PPARγ protein abundance was not significant reduced in preterm lambs managed by NIS+ES compared to NIS. The minimal reduction in PPARγ protein abundance could be attributed to feeding tolerability and an increase in fatty acid substrate availability. However, FABP2 protein abundance was significantly altered in preterm lambs managed by NIS+ES compared to preterm lambs managed by NIS. In contrast, occludin protein abundance appears to be greater in NIS+ES than preterm lambs managed by NIS. A biological advantage in preterm lambs managed by NIS+ES to promoting the transcription of occludin would be to decrease paracellular permeability. Sedation interference with motility, coupled with increase permeability, would increase the risk bacteria translocation, resulting in systemic inflammatory responses and sepsis[2].

Our data suggest that PPARγ and FABP2 may be affected by altered circulating fatty acids due to restricted nutrition. In preterm neonates, change in long-chain fatty acid levels during the first postnatal month is linked to comorbidities such as chronic lung disease and late-onset sepsis in human neonates[31]. However, clinical trails of long-chain polyunsaturated fatty acid supplementation for NEC in preterm neonates have not shown a reduction in NEC[32]. These studies were not looking at NEC as the primary outcome measure and lacked power[32]. Continuing research in our lab is being conducted on DHA supplementation in preterm lambs managed by IMV and NIS+RN to validate that poor postnatal nutrition secondary to prematurity is a causal factor involved in the regulation of the PPARγ and FABP2 expression and poor gut integrity. Currently, our lab has a pilot experiment to examine the effects of daily DHA supplementation of 150 mg/kg/d on the

development of NEC-like phenotype, preterm lambs managed by IMV for 21 days.

Our preterm lamb model is unique in that damage to the intestines is due to natural responses to prematurity, mode of ventilation and poor postnatal nutrition. Most animal models are designed to examine the molecular mechanisms of NEC by induced damage via hypoxia-ischemia or formula feeding. These methods may not accurately recapitulate the nature of human NEC. A limitation to our study is a small sample size. Sample size is limited due to the high cost of generating and managing the preterm lambs, particularly for the 21 days studies. Our experiments were also not powered to determine differences in outcome between male and female preterm lambs for any interventions. Additionally, inflammation is a factor in the development of NEC and in our research, we have not evaluated inflammatory markers in any lamb groups at this time. A further limitation of our study is that it is descriptive. We have not assessed cause-and-effect relationships between PPARγ signaling and gut integrity.

The morbidity and mortality rate of NEC remains unchanged, largely because of the lack of understanding of pathogenic pathways and protective measures. Elucidating the molecular mechanism of PPARγ protection on intestinal integrity through interactions with FABP2 will provide support for PPARγ-pathway activation as a targeted therapeutic. If supplementation of DHA in preterm lambs managed by IMV and NIS+RN improve feeding tolerance and gut integrity, marked by increased PPARγ and FABP2 expression, it would support early introduction of PPARγ agonist as a protective and a preventative treatment for NEC.

In conclusion, our results demonstrated that IMV, NIS+RN and NIS+ES decrease PPARγ and FABP2 expression in the preterm lamb ileum. Disruption to PPARγ and

FABP2 expression by IMV and inadequate nutrition likely has detrimental effects on gut integrity and may contribute to a NEC-like phenotype. Further research on occludin expression is needed to further understand the association of PPARγ and occludin.

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